Postharvest Biology and Technology 88 (2014) 54–60

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Increasing maturity reduces wound response and lignification processes against *Penicillium expansum* (pathogen) and *Penicillium digitatum* (non-host pathogen) infection in apples

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ARTICLE INFO

Article history:
Received 18 June 2013
Received in revised form
16 September 2013
Accepted 23 September 2013

Keywords:
Blue mould
Green mould
Maturity stage
Wound response
Lignin
Defence

ABSTRACT

Penicillium expansum is the main postharvest pathogen of pome fruit and is a necrotrophic fungus that requires wounds to infect the fruit. Therefore, injuries caused during harvest and postharvest handling provide an optimal locus for infection. In this study, the effect of wound response in apples harvested at three different maturity stages and stored at two different temperatures (20 and 0 °C) infected with *P. expansum* (pathogen) and *Penicillium digitatum* (non-host pathogen) was evaluated. The effect of wounding and pathogen inoculation on lignin content was also quantified. At 20 °C, less decay incidence and severity were observed when time between wounding and inoculation increased, and these differences were more important in fruit from immature and commercial harvests. However, at 0 °C, wound response was too slow to prevent *P. expansum* infection. Lignin content was highest in fruit from the immature harvest. Our results indicated that maturity and storage temperature play an important role in apple wound response. This is the first report demonstrating that *P. digitatum*, a non-host pathogen, was able to develop rots in over-mature apples.

1. Introduction

Penicillium expansum causes blue mould disease in a broad range of hosts, including apples, and is one of the most destructive pathogens of pome fruit, reaching up to 50% of stored fruit losses (Mari et al., 2002). This pathogen is a necrotroph and requires a wound in the epidermis to enter fruit tissue and initiate infection (Spotts et al., 1998). Conidia of *Penicillium* species are ubiquitous in the atmosphere of packinghouses (Barkai-Golan, 1966), even in production areas where the most advanced storage technologies are used (Conway et al., 2004; Spadaro et al., 2004). Therefore, mechanical injury caused during harvesting and postharvest handling provides an optimal locus for infection. Moreover, biochemical changes associated with fruit ripening such as cell wall breakdown and membrane alteration, increase susceptibility to mechanical damage and may favour the infection process (Cantu et al., 2008). Although control of this important pathogen can be achieved by using chemical fungicides, the growing concern for human and environmental health risks associated with pesticide usage, the development of fungicide-resistant strains, and the lack of approval of some of the most effective fungicides, have motivated the search for alternative approaches. The most promising alternative strategies being developed are mainly based on heat application, sodium bicarbonate, hot water dipping and biological control with antagonistic microorganisms (Janisiewicz and Korsten, 2002; Mari et al., 2003; Spadaro and Gullino, 2004; Droby et al., 2009; Teixidó et al., 2011). However, very few studies have been conducted in fruit to elucidate the host-pathogen interaction in order to characterize the innate resistance of apples against *P. expansum*.

Plants in general, and fruit in particular, can defend themselves against pathogens by genetically determined defence mechanisms, expressed either constitutively (pre-occurring barriers as waxy cuticles and cell walls) or induced as a consequence of a biotic or abiotic factors. The early phase of plant response to wounding is critical (Gayoso et al., 2010) because a rapid and efficient deployment of defence responses can prevent further pathogen invasion (Su et al., 2011) limiting pathogen establishment and colonization (Ferreira et al., 2006). Wound responses in plants have been extensively studied (Leon et al., 2001; Schilmiller and Howe, 2005), and it has been hypothesized that plants have evolved mechanisms that integrate both pathogen-specific and general wounding responses (Castro-Mercado et al., 2009). Wounding regulates a number of genes that are associated with a pathogen-specific response (Durrant et al., 2000; Reymond et al., 2000), indicating that innate and pathogen-specific responses share a number

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http://dx.doi.org/10.1016/j.postharvbio.2013.09.009

of components in their signalling pathways (Maleck and Dietrich, 1999).

One of the earliest events that is widely induced in response both to wounding and to pathogen attack is a rapid generation of reactive oxygen species (ROS) during the so-called oxidative burst (Bradley et al., 1992; Orozco-Cardenas et al., 2001; Bindschedler et al., 2006). In addition to its oxidative potential in killing or inhibiting the growth of pathogens, ROS production has been associated with the formation of physical defensive barriers around wounds (Huckelhoven and Kogel, 2003) involving the formation of glycoproteins, callose, lignin, and other phenolic polymers (Lamb and Dixon, 1997). Lignification occurs through a series of enzymatic steps involving the phenylpropanoid pathway, a pathway that generally contributes to a variety of plant responses to biotic and abiotic stimuli (Vogt, 2010).

The wound healing response results in the production of wound periderm, which was thought to be lacking in fruit after harvest (Skene, 1981). However, Lakshiminarayana et al. (1987) showed an accumulation of phenolics and lignin-like materials around wounds in mature fruit and an effective protection from pathogen invasion. More recent studies by Vilanova et al. (2012a) showed lower defence response in over-mature apples, demonstrating that maturity stage of fruit is an important factor in apple defence response. However, some questions remain unanswered such as how apple wound response may affect resistance against pathogens.

The aim of the present study was to investigate apple wound response to compatible (*P. expansum*) and non-host (*Penicillium digitatum*) pathogens at different maturity stages and storage temperatures. The wound response studies were combined with biochemical analysis in order to define the role of lignin content in host resistance against both pathogens.

2. Materials and methods

2.1. Fruit

'Golden Smoothee' apples (*Malus* × *domestica* Borkh.) were obtained from a commercial orchard in Mollerussa (Catalonia, Spain) and used immediately after harvest. Harvests were carried out on 16 August (harvest 1), 16 September (harvest 2) and 22 October (harvest 3), 2010. Harvest 1 was considered as prior to commercial maturity (immature harvest), harvest 2 was considered commercial maturity (commercial harvest) and harvest 3 was considered past maturity (over-mature harvest). Fruit were selected for uniform size, without physical injuries or apparent infections. Once the apples arrived at the laboratory, they were surface disinfected with 10% sodium hypochlorite for 1 min, rinsed with tap water, and allowed to dry at room temperature.

2.2. Determination of quality parameters

Colour, firmness, starch index, soluble solids, and acidity were determined as quality parameters at each harvest date. Colour was measured using hue values, which were calculated from a^* (red-greenness) and b^* (yellow-blueness) values measured with a CR-200 chromameter (Minolta, Japan) on both the exposed and shaded sides of each fruit, using standard CIE illuminant and 8 mm viewing aperture diameter. Flesh firmness was measured on two opposite sides of each fruit with a penetrometer (Effegi, Milan, Italy) equipped with an 11 mm diameter plunger tip. Starch hydrolysis was rated visually using a 1-10 EUROFRU scale (1, full starch; 10, no starch) (Planton, 1995), after dipping of cross-sectional fruit halves in 0.6% (w/v) I_2–1.5% (w/v) KI solution for 30 s. Total soluble solids content (TSS) and titratable acidity (TA) were assessed in juice using a refractometer (Atago, Tokyo, Japan) and by titration of 10 mL of juice with 0.1 N NaOH and 1% phenolphthalein as an indicator. Data on maturity indexes represent the means of 20 individual fruit.

2.3. Fungal cultures

P. expansum (CMP-1) and *P. digitatum* (PDM-1) are the most aggressive isolates in our collection of isolates capable of infecting pome and citrus fruit, respectively. They are maintained on potato dextrose agar medium (PDA; 200 mL boiled potato extract, 20 g dextrose, 20 g agar and 800 mL water) and periodically grown on wounded apples (*P. expansum*) or oranges (*P. digitatum*) and then re-isolated to maintain virulence. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01% (w/v) Tween-80 over the surface of 7- to 10-d-old cultures grown on PDA and rubbing the surface of the agar with a sterile glass rod. Conidia were counted in a haemocytometer and diluted to the desired concentration.

2.4. Wound response studies

The effect of maturity and storage temperature on wound response was assessed for both the compatible interaction (*P. expansum*-apples) and the incompatible interaction (*P. digitatum*-apples). Apples were wounded once with a nail (1 mm wide and 2 mm deep). To evaluate the effect of storage temperature on wound response, fruit were separated in two different sets; one was stored at 20 °C and the other at 0 °C.

Fruit stored at 20 °C were divided into 7 different subgroups, each one inoculated at different times after wounding: time 0 h (wounded and inoculated at the same time) served as a control while the other 6 subgroups were inoculated at 1, 2, 3, 4, 7 or 10 d after wounding. The experiment was carried out for each pathogen and at each maturity stage. In all cases, fruit were inoculated with 15 μL aqueous conidia suspensions of *P. expansum* at 10^4 conidia mL^{-1} and *P. digitatum* at 10^7 conidia mL^{-1}. Incidence and severity of lesions were evaluated after 7, 10 and 15 d of inoculation for each pathogen, time between wounding and inoculation, and maturity stage.

Fruit stored at 0 °C were divided into 5 different subgroups, each one inoculated at different times after wounding: time 0 h (wounded and inoculated at the same time) served as a control while the other 4 subgroups were inoculated at 4, 7, 15 or 30 d after wounding. Fruit were inoculated as previously described and the experiment was carried out for each pathogen and maturity stage. Incidence and severity of lesions were evaluated at 60, 90 and 120 d after inoculation for each pathogen, time between wounding and inoculation, and maturity stage.

In both cases (20 °C and 0 °C), five apples constituted a single replicate and each treatment was replicate four times.

2.5. Lignin studies

Lignin content of apples was measured at three different maturity stages and at four times after inoculation (24 h, 48 h, 72 h and 7 d) for both pathogens.

To measure the lignin content, twenty wounds were made on one side of each apple with a nail in a manner similar to that used in the wound response studies, and inoculated with 10 μL of aqueous conidial suspensions of either *P. expansum* or *P. digitatum* at 10^5 and 10^7 conidia mL^{-1}, respectively. Control fruit were wounded and inoculated with 0.01% (w/v) Tween-80. Fruit were stored at 20 °C and 85% RH for 24, 48, 72 h, and 7 d.

After each storage time, fifteen cylinders of apple tissue (8 mm inside diameter and 3 mm deep containing peel and pulp) encompassing the wounds were removed from each apple using a cork

borer. Sixty disks from four fruit were pooled and considered a single replicate and three replicates were evaluated for each sample collection.

The estimation of lignin content was performed according to Nafussi et al. (2001) with slight modification. Briefly, frozen apple disks were lyophilized for 4 d and then ground to a fine powder. Each sample was sequentially washed with water, ethanol, acetone and diethyl ether through Whatman 1 filter paper until the washed tissue was colourless. The resulting powder was dried at 70 °C for 1 h, and 20 mg samples were digested with a solution of 25% (w/w) acetyl bromide in acetic acid (2.5 mL) and $HClO_4$ (70%, 0.12 mL) and heated in a bath at 70 °C for 30 min with shaking. After cooling with ice, 10 mL of 2 M NaOH and 12 mL of acetic acid were added to the reaction tubes and 1.5 mL of the resulting solution was centrifuged at 14,000 × *g* (Mikro 22R, Hettich Zentrifugen, UK) for 11 min at room temperature to be sure that the resulting sample was completely clear. Each solution was diluted 5 times with acetic acid and absorbance was measured at 280 nm. For each replicate, three technical measurements were done.

2.6. Data analysis

Data regarding incidence and severity of decayed fruit, lignin content and quality parameters were analyzed for significant differences by analysis of variance (ANOVA) with JMP 8 (SAS Institute Inc., NC, USA) statistical package. Before analysis of data expressed as percentages, homogeneity of variance was tested by Barlett's test and data were transformed to the arcsine of the square root. Statistical significance was deemed when $P < 0.05$. When the analysis was statistically significant, a Tukey test for separation of means was performed.

3. Results

3.1. Effect of maturity stage and time between wounding and inoculation on development of blue mould caused by P. expansum

In general, at 20 °C the elapsed time between wounding and inoculation had a significant effect on restricting *P. expansum* infection and the effect was more pronounced in fruit from the immature and commercial harvests compared to the over-mature harvest. The effect on restricting *P. expansum* infection obtained at 0 °C was less intense than at 20 °C.

3.1.1. At 20 °C

Differences among times between wounding and inoculation at 7 and 10 d were difficult to evaluate because of the small lesion size (less than 2 cm) that occurred at immature and commercial harvests (data not shown). Fig. 1 represents observations made at 15 d following inoculation.

In fruit from the immature harvest, decay incidence was approximately 40% when the inoculation was delayed to 1 d after wounding (Fig. 1A). Disease incidence in fruit inoculated at time 0 h after wounding was 100%, therefore the delay in inoculation represented approximately a 60% reduction in disease incidence. When fruit were inoculated at 2 and 3 d after wounding, decay incidence was reduced approximately 75 and 90%, respectively. No rot development was found when fruit inoculation was delayed 4 or more d after wounding. In fruit from commercial harvest, when the inoculation was delayed 1 and 2 d after wounding, decay incidence was reduced approximately 60% and 70%, respectively. When inoculation was delayed 3 d or longer, decay incidences were around 5%. In fruit from the over-mature harvest, decay incidence was not reduced to 50% until fruit were inoculated at 10 d after wounding.

A comparison among fruit from different harvests showed no significant differences in decay incidence when they were inoculated at 0 h after wounding (Fig. 1A). However, fruit from immature and commercial harvests inoculated at 1, 2, 3, 4, 7 and 10 d after wounding showed lower incidence (around 40%, 30%, 7%, 3%, 3% and 3%, respectively) than fruit from the over-mature harvest (95%, 90%, 93%, 76%, 79% and 50%, respectively).

Within each harvest time, lesion diameter decreased as elapsed time between wounding and inoculation increased (Fig. 1B). Fruit from the immature harvest inoculated at 0 h after wounding showed the largest lesion diameter (around 5 cm) in comparison to fruit inoculated at 1, 2, 3, 4, 7 and 10 d after wounding (1, 0.5, 0.1, 0, 0 and 0 cm, respectively). At commercial harvest, the effect of elapsed time between wounding and inoculation on lesion diameter showed three statistically different groups (0 h; 1–2 d; 3–10 d after wounding) with lesion diameters around 7, 0.7 and 0.05 cm, respectively. Fruit from the over-mature harvest inoculated at 7 and 10 d after wounding showed smaller lesion diameters (4.3 and 2.7 cm, respectively) compared to those inoculated at 0 h after wounding (7 cm).

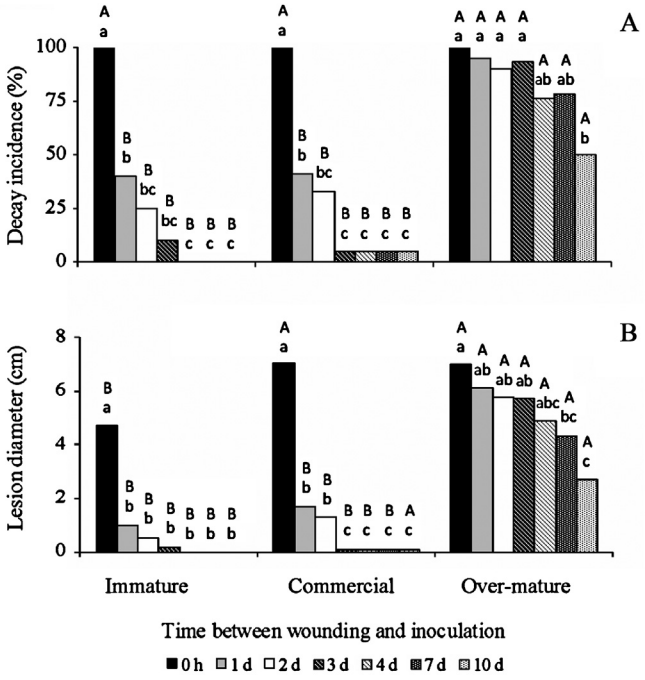

Fig. 1. Disease incidence (A) and lesion diameter (B) in 'Golden Smoothee' apples harvested at three maturity stages and inoculated with *Penicillium expansum* at different times after wounding and stored at 20 °C and 85% RH for 15 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 apples.

Fruit from the immature harvest inoculated at 0 h after wounding had smaller lesion diameters (4.7 cm) than those from either the commercial and over-mature harvests (7 cm) (Fig. 1B). Fruit from immature and commercial harvests inoculated at 1, 2, 3, 4, 7 and 10 d after wounding showed lower lesion diameters (around 1.3, 0.9, 0.2, 0, 0 and 0 cm, respectively) than fruit from the over-mature harvest (6.1, 5.7, 5.7, 4.8, 4.3 and 2.7 cm, respectively).

3.1.2. At 0 °C

Observations made at 60 d after inoculation were difficult to evaluate because of the small lesion size (less than 1 cm) that occurred (data not shown), and at 120 d after inoculation, differences among times between wounding and inoculation were difficult to evaluate because most of apples were completely rotten (data not shown). Fig. 2 represents observations made at 90 d after inoculation.

A comparison among fruit from different times between wounding and inoculation and from different harvests showed no significant differences in decay incidence (Fig. 2A). However, in fruit from the immature harvest, there was a distinct tendency for the later inoculation times to exhibit reduced incidence since fruit inoculated at 0 h, 4 and 7 d after wounding showed around 100% decay incidence and those inoculated at 15 and 30 d after wounding showed 80% and 65% decay incidence, respectively.

Fruit from the immature harvest inoculated at 30 d after wounding had smaller lesion diameters (0.6 cm) in comparison to fruit inoculated at 0 h after wounding (1.4 cm) (Fig. 2B). No significant differences in lesion diameters were found when immature fruit were inoculated at 0 h, 4, 7 and 15 d after wounding. Fruit from the commercial harvest inoculated at 7 d after wounding or longer had lower lesion diameters (around 2 cm) in comparison to fruit inoculated at 0 h after wounding (around 3.3 cm). However, no differences between times after wounding were found at the over-mature harvest.

At short times between wounding and inoculation (0 h and 4 d), immature harvest fruit had smaller lesion diameters than those from the commercial harvest (3.3 and 2.9 cm) and those from the over-mature harvest (3.9 and 3.4) (Fig. 2B). However, when fruit were inoculated at 15 and 30 d after wounding, there were differences in lesion diameter among three harvests, and lesion diameters increased with fruit maturity.

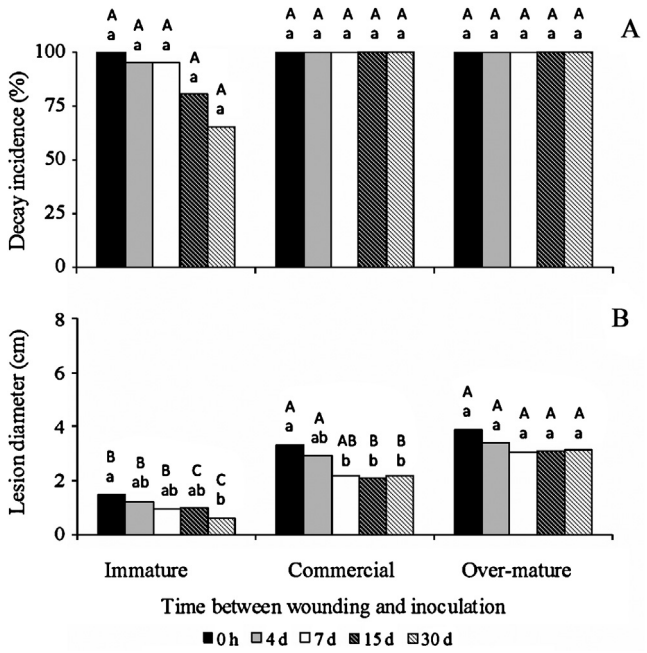

Fig. 2. Disease incidence (A) and lesion diameter (B) in 'Golden Smoothee' apples harvested at three maturity stages and inoculated with *Penicillium expansum* at different times after wounding and stored at 0 °C and 85% RH for 90 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 apples.

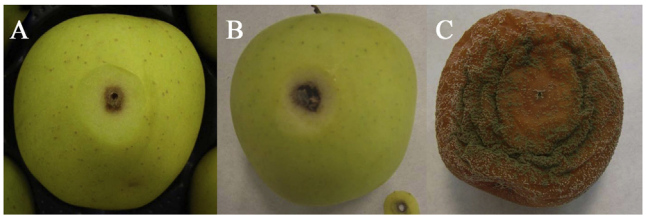

Fig. 3. 'Golden Smoothee' apples inoculated with *Penicillium digitatum*. A visible reaction around the inoculation site was found in immature apples (A). *P. digitatum* was able to infect a small group of apples from commercial harvest (B) but was not able to develop decay. *P. digitatum* was able to infect and develop rot on apples from over-mature harvest reaching a complete apple rot at 10 d after inoculation (C).

3.2. Effect of maturity stage and time between wounding and inoculation on development of mould caused by P. digitatum

P. digitatum only was able to develop rots on over-mature apples stored at 20 °C. Interestingly, when *P. digitatum* can overcome apple defences, rot development progressed very fast, reaching a complete apple rot at 10 d after inoculation (Fig. 3C).

3.2.1. At 20 °C

No decay symptoms were observed in fruit from the immature harvest (Fig. 3A); however, a small number of commercial apples showed infection but the decay was limited to the initial infection site (Fig. 3B). A prominent reaction was observed in the peel and in the pulp (dead tissue) of the fruit when *P. digitatum* did not infect apples. That reaction was most prominent at immature than at commercial harvest. No reaction appeared at over-mature harvest.

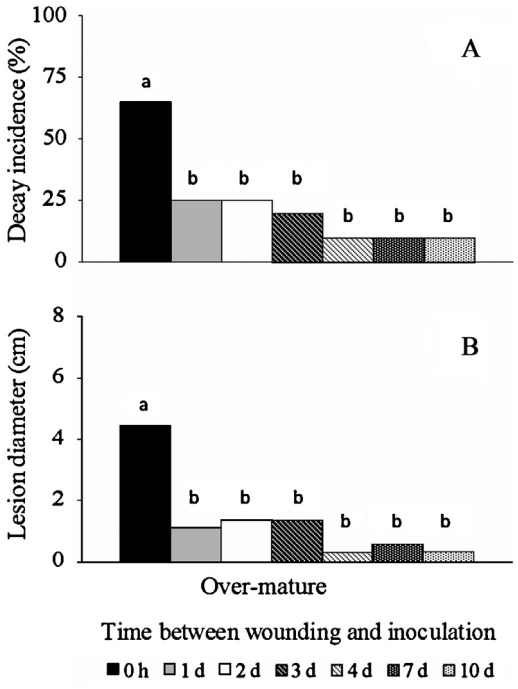

Fig. 4. Disease incidence (A) and lesion diameter (B) in 'Golden Smoothee' apples harvested at over-mature stage and inoculated with *Penicillium digitatum* at different times after wounding and stored at 20 °C and 85% RH for 10 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. Letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). Each column represents the mean of 20 apples.

In general, little evidence of decay was present at 7 d after inoculation (data not shown) and at 15 d after inoculation, the results obtained were very similar to those obtained at 10 d after inoculation. Fig. 4 represents observations made at 10 d after inoculation.

Decay incidence in over-mature apples inoculated with *P. digitatum* was approximately 25% when the inoculation was delayed 1 or 2 d after wounding (Fig. 4A). Disease incidence in fruit inoculated at time 0 h after wounding was 65%, therefore the delay in inoculation was higher than 75% in disease incidence. Lesion diameter also significantly decreased when the inoculation was delayed 1 or more d (Fig. 4B). Over-mature apples inoculated at 0 h after wounding showed the largest lesion diameter (4.5 cm) in comparison to fruit inoculated at the different times after wounding (less than 2 cm).

3.2.2. At 0 °C

At 0 °C storage temperature, *P. digitatum* was not able to develop infection regardless of maturity stage of fruit. The reaction behaviour was the same as that obtained at 20 °C but the reaction was less intense (data not shown).

3.3. Apple quality parameters

There were significant decreases in titratable acidity and flesh firmness as the harvest date progressed (Table 1). In contrast, total soluble solids, starch index and ($a^* + b^*$) values were higher with later harvest date.

3.4. Lignin content

When the samples showed decay (independently of whether they were inoculated with *P. expansum* or *P. digitatum*), the absorbance values obtained at 280 nm as lignin content were unusually high (data not shown) and may be due to cell wall degradation by fungi (Vilanova et al., 2013).

Table 1
Effect of harvest date on fruit quality parameters of 'Golden Smoothee' apples. Values for harvest dates with the same letter are not significantly different ($P < 0.05$) according to the Tukey test.

Harvest	Date	Total soluble solids (TSS in %)	Titratable acidity (g L^{-1} malic acid)	Flesh firmness (N)	Starch index	($a^* + b^*$)
Immature	16/08/2010	10.2c	6.3a	77.9a	1.1c	23.1b
Commercial	16/09/2010	12.1b	6.1a	65.9b	6.0b	24.4b
Over-mature	22/10/2010	14.2a	4.3b	42.8c	9.8a	39.9a

Table 2
Lignin content (absorbance at 280 nm) of 'Golden Smoothee' apples wounded and inoculated with water (control), *P. digitatum*, or *P. expansum* and stored at 20 °C for different periods of time. Apples were harvested at three different maturity stages. For each harvest, times after inoculation with different lowercase letters are statistically different according to the Tukey test ($P < 0.05$). For each time after inoculation, harvests with different uppercase letters are statistically different according to Tukey test ($P < 0.05$).

		Lignin content (absorbance at 280 nm)		
Harvest	Time after inoculation	Control	*P. digitatum*	*P. expansum*
Immature	24 h	0.2997aA	0.2992bA	0.3954aA
	48 h	0.3082aA	0.3532abA	0.3822aA
	72 h	0.2927aA	0.3770aA	Rot develop
	7 d	0.3322bA	0.3478abA	Rot develop
Commercial	24 h	0.2006aB	0.2168bB	0.2055aB
	48 h	0.2166aB	0.2172bB	0.2077aB
	72 h	0.2038aB	0.2455aB	Rot develop
	7 d	0.2039aB	0.2586aB	Rot develop
Over-mature	24 h	0.2324aB	0.2333B	0.2405B
	48 h	0.2372aB	Rot develop	Rot develop
	72 h	0.2378aB	Rot develop	Rot develop
	7 d	0.2393aB	Rot develop	Rot develop

Differences in lignin content with time after inoculation were evident in the control treatment in fruit from the immature harvest and in samples inoculated with *P. digitatum* in fruit collected from the immature and commercial harvests (Table 2). In general, the most important differences between times after inoculation were observed in fruit inoculated with *P. digitatum* at immature and commercial harvests.

A comparison among fruit from different harvests showed higher lignin contents at immature harvest than at commercial and over-mature harvests for each of the assayed treatments (Table 2). At immature harvest, fruit inoculated with *P. expansum* had higher lignin contents than controls or fruit inoculated with *P. digitatum*. However, at commercial harvest, differences among treatments were only observed from 72 h after inoculation, with higher lignin contents in fruit inoculated with *P. digitatum* than in control fruit.

4. Discussion

The current study evaluated wound response in 'Golden Smoothee' apples in relation to the ability of *P. expansum* (pathogen) and *P. digitatum* (non-host pathogen) to infect fruit at different maturity stages and storage conditions. This is the first work that reports the capacity of *P. digitatum* (non-host pathogen) to develop rots in apples under specific conditions.

Apple wound response had a significant effect on restricting *P. expansum* infection when fruit were stored at 20 °C. Immature and commercial apples presented an important decrease in both disease incidence and lesion diameter when inoculation was delayed 3 d after wounding. Su et al. (2011) also found a decrease in disease incidence in early and optimal harvested 'Gala' apples when *Botrytis cinerea* inoculation was delayed 96 h after wounding. Shao et al. (2010) obtained similar disease incidence when commercial 'Gala' apples were inoculated with *P. expansum* 96 h after wounding. In pears, Spotts et al. (1998) found that wound response decreased the susceptibility of wounds to *P. expansum* infection after 2 d at 20 °C. In oranges, Vilanova et al. (2013) observed similar reductions when the inoculation was delayed more than 4 d. Wound response appears to be more efficient in apples and in pears than in oranges in providing resistance to a compatible pathogen. Green peppers showed the most effective wound response in resistance against *Colletotrichum acutatum*, resulting in a great reduction in lesion size when the inoculation was delayed only 1 h after wounding (Kim et al., 2008).

The effect of apple wound response on resistance showed a temperature dependent behaviour. Results indicated that wound response at cold temperatures was insufficient to prevent colonization of *P. expansum*. Temperatures and relative humidity are the two most important conditions affecting the wound healing process and generally, temperatures above 10 °C and a relative humidity above 85% are required (Brown, 1989). In apples (Skene, 1981; Vilanova et al., 2012a) and also in oranges (Vilanova et al., 2013) wound-healing processes at 20 °C were more active than at 0 °C. Moreover, *P. expansum* is mainly a cold storage condition pathogen, and Buron-Moles et al. (2012) showed that 90% of spores germinated within 6 d at 0 °C. However, Lakshiminarayana et al. (1987) observed a strong resistance in apples inoculated with *B. cinerea* and *P. expansum* within 4 d after wounding at 5 °C, although they attribute the resistance response to processes other than wound healing because they considered that 4 d between wounding and inoculation was not enough time to produce modification in the cell wall at 5 °C. Spotts et al. (1998) reported that pears stored at −1 °C and inoculated 28 d after wounding decreased decay incidence from 93% to 35 %.

Although *P. digitatum* is a very specific pathogen that only infects citrus fruit, in this work we surprisingly found that the fungus was able to develop rots in over-mature apples. Vilanova et al. (2012a) showed the capacity of *P. digitatum* to infect apples from commercial harvest but the decay was limited to initial infection site. Thus it seems that when *P. digitatum* can overcome apple defences, it is able to develop almost as fast as the compatible pathogen. Macarisin et al. (2007) in oranges showed the capacity of *P. expansum* (non-host) pathogen to germinate and temporarily grow at 20 °C. However, more recent studies from Vilanova et al. (2012b, 2013) showed that *P. expansum* could develop rot in oranges under specific conditions.

Despite the apple response being less active at 0 °C, no signs of *P. digitatum* infection were found in any of the conditions studied, as previously shown by Vilanova et al. (2012a). These results could be explained by *P. digitatum* being able to germinate and grow in the range 4–30 °C and the germination delaying and slowing when the temperature decreased (Plaza et al., 2003). Different behaviour was obtained by Vilanova et al. (2012b) in the incompatible interaction *P. expansum*-oranges that showed higher decay incidence and severity at 4 °C than at 20 °C. These results could be explained because *P. expansum* in contrast to *P. digitatum* is well adapted to cold temperatures (Gougouli and Koutsoumanis, 2010).

The wound healing response resulted in the production of wound periderm (Skene, 1981), which was associated with a local accumulation of phenolics and lignin in cell wall thickening around wounds (Lakshiminarayana et al., 1987; Spotts et al., 1998). Different authors have correlated wound healing processes in apples with lignin accumulation using qualitative (Lakshiminarayana et al., 1987; Vilanova et al., 2012a) and quantitative methods (Valentines et al., 2005; Shao et al., 2010; Su et al., 2011). Numerous methods have been developed over the past years to measure lignin levels in different plant species (Hatfield and Fukushima, 2005), however, choosing the most suitable method for each fruit remains a difficult task. In this study the acetyl bromide method reported by Nafussi et al. (2001) was adapted to apple fruit. Our results

showed that control apples from the immature harvest increased in lignin content with storage time, with the highest lignin quantity at 7 d after inoculation. Similar results were reported by Su et al. (2011) in 'Gala' apples where a greater increase in lignin was observed in wounded tissue from early harvested compared to late harvest fruit. Valentines et al. (2005) found higher lignin contents in more resistant apples to *P. expansum* infection which means that lignin accumulation also plays an important role in apple defence response against pathogens. Vilanova et al. (2012a) also found a positive lignin reaction in wounded immature apples while the reaction was negative in commercial and over-mature apples.

The present study demonstrated an increase in lignin content in immature and commercial apples inoculated with *P. digitatum* (non-host pathogen), with the highest lignin content being from 72 h post-inoculation. These results are in agreement with those obtained by Vilanova et al. (2012a) in apples and Romero et al. (2008) in melon leaves. Immature apples inoculated with *P. expansum* also had higher lignin contents, as previously shown by Vilanova et al. (2012a). It seems that apples defend more intensely against the compatible than the incompatible pathogen. However, *P. expansum* is able to overcome these defences and develop infection.

This study offers information about how apple wound response can be affected by both maturity stage and storage conditions (temperature), providing a pathological and biochemical approach of apple wound response. The wound response process at 20 °C can prevent infection by both compatible, *P. expansum* and non-host, *P. digitatum*, pathogens. However at cold temperatures, wound response was too slow to prevent *P. expansum* infection. Additionally, wound response declined with fruit ripening, which resulted in increased wound susceptibility to both *P. expansum* and *P. digitatum*. Lastly, we demonstrated that lignin could function as a defence mechanism against *P. expansum* and *P. digitatum*. However, that reaction cannot prevent *P. expansum* development. Until now, *P. digitatum* was considered as a non-host pathogen of apples, but in this study, it has been demonstrated that it can develop rots in over-mature apples.

Acknowledgments

We thank Robert Oró for his excellent technical assistance. The authors are grateful to the Spanish Government for financial support by two national projects AGL2008-04828-C03/AGR and AGL2011-30519-C03/AGR (Plan Nacional de I + D + I, Ministerio de Ciencia e Innovación, Spain) and the 'Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria' (INIA) for L. Vilanova PhD grant.

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